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Nrf2 regulates *NGF* mRNA induction by carnosic acid in T98G glioblastoma cells and normal human astrocytes

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Nerve growth factor (NGF) is a neurotrophic factor that plays an important role in neuronal cell development and survival. Carnosic acid (CA), a hydrophobic constituent of the herb rosemary, induces NGF production in human T98G glioblastoma cells, but the mechanism through which it works remains unknown. In the present study, we found a redox-sensitive transcription factor, Nrf2, which coordinates the expression of cytoprotective phase 2 genes, also participates in CA-inducible *NGF* expression. In T98G cells, CA caused *NGF* gene induction in a dose- and time-dependent manner without altering *NGF* mRNA stability. Simultaneously, CA increased Nrf2 nuclear accumulation and activated expression of prototypical Nrf2 target genes such as haem oxygenase 1 (*HO-1*) and thioredoxin reductase 1 (*TXNRD1*). Knockdown of endogenous Nrf2 by Nrf2-specific siRNA significantly reduced constitutive and CA-inducible *NGF* gene expression. In addition, *NGF* gene expression was enhanced by knockdown of Keap1, an Nrf2 inhibitor, in the absence of CA. Furthermore, CA induced *NGF* expression in normal human astrocytes in an Nrf2-dependent manner. These results highlight a role of Nrf2 in *NGF* gene expression in astroglial cells.

Keywords: astrocyte/carnosic acid/NGF/Nrf2/oxidative stress.

Abbreviations: ARE, antioxidant responsive element; BDNF, brain-derived neurotrophic factor; CA, carnosic acid; CNC, Cap'n'Collar; bZip, basic leucine zipper; DMSO, dimethyl sulphoxide; GCLC, glutamate-cysteine ligase catalytic subunit; HO-1, haem oxygenase 1; IL-1β, interleukin-1β: Keap1, kelch-like ECH-associated protein 1; NGF, nerve growth factor; NHAs, normal human astrocytes; Nrf2, NF-E2-related factor 2; NT-3, neurotrophin-3; SQSTM1, sequestosome 1; TNFα, tumour necrosis factor α; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; TXNRD1, thioredoxin reductase 1.

Nerve growth factor (NGF) is a member of the neurotrophin family, which includes brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (*1*, *2*). NGF plays an important role in the development of peripheral sensory and sympathetic neurons as well as in the maintenance of cholinergic neurons of the adult forebrain (*1–3*) by binding to a cell surface tyrosine kinase receptor consisting of $p140^{TrkA}$ and $p75^{NTR}$ (*4*). Because of its neuroprotective effect, NGF has been explored as a therapeutic target for ischaemic neuronal cell death and neurodegenerative diseases, such as Alzheimer's disease (*2*). However, because exogenous NGF cannot cross the blood–brain barrier (BBB), compounds that can permeate the BBB and activate NGF production are promising therapeutic candidates for neurodegenerative diseases. NGF is produced by both neurons and glial cells, such as astrocytes and oligodendrocytes. In addition, non-neuronal cells, such as monocytes, macrophages, keratinocytes and fibroblasts, also produce NGF. In these cells, NGF production is regulated both transcriptionally and post-transcriptionally. Okadaic acid and IL-1β induce NGF expression by increasing both mRNA stabilization and gene transcription (*5–7*). On the other hand, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the β-adrenergic receptor agonist clenbuterol (CLE), tumour necrosis factor α (TNF-α), basic fibroblast growth factor and lipopolysaccharide induce *NGF* mRNA transcription (*6*, *8–13*). However, the precise molecular mechanisms of *NGF* gene regulation are not fully understood.

NF-E2-related factor 2 (Nrf2) is a CNC-bZip transcription factor that plays a key role in redox regulation and drug metabolism (*14*, *15*). Nrf2 is activated by reactive oxygen species (ROS) and exogenous and endogenous electrophiles, such as sulphoraphane, 6-methylsulfinylhexyl isothiocyanate and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). In the absence of stimuli, Nrf2 is trapped by the Kelch-like ECH-associating protein 1 (Keap1)-Cul3-Rbx1

complex and constitutively degraded through the ubiquitin–proteasome pathway (*14–16*). Under oxidative stress or in the presence of electrophiles, Keap1 is inactivated by oxidative modification of its cysteine residues, allowing for Nrf2 stabilization and translocation into the nucleus. In the nucleus, Nrf2 heterodimerizes with small Maf proteins (sMaf), and the Nrf2/sMaf heterodimer binds to a DNA *cis*-element which is called an antioxidant responsive element (ARE) or electrophile responsive element (EpRE) (*14*, *15*). AREs are found in the regulatory regions of phase 2 enzyme genes, such as haem oxygenase 1 (*HO-1*); NAD(P)H quinone oxidoreductase 1 (*NQO1);* glutamate–cysteine ligase, catalytic subunit (*GCLC*); thioredoxin reductase 1 (*TXNRD1*) and glutathione *S*-transferases (*GSTs*). Nrf2 activation by electrophiles decreases oxidative stress-induced toxicities and prevents carcinogen-induced tumourigenesis in various organs (*14*, *17*, *18*). Targeted disruption of *Nrf2* gene in mice increases their susceptibility to oxidative stress and chemical-induced carcinogenesis (*18*, *19*). In the brain, Nrf2 functions as a neuroprotective factor against oxidative stress and excitotoxicity (*20*, *21*). The neuroprotective effects of Nrf2 derive from both the neuronal and astrocytic functions of Nrf2 (*21*).

Carnosic acid (CA) is a phenolic diterpene found in the dietary herb rosemary (*Rosmarinus officinalis* L.), and it exerts its antioxidant properties by serving as a radical scavenger (*22*, *23*). In addition to its antioxidant function, CA functions as a peroxisome proliferator-activated receptor γ (PPARγ) agonist or a 5-lipoxygenase inhibitor in mammalian cells (*24*, *25*). Moreover, we recently found that CA activates the Nrf2 signalling pathway in neuronal cells through *S*-alkylation of Keap1 cysteines (*26*). CA can cross the BBB and attenuate middle cerebral artery occlusion (MCAO)-induced neuronal cell death by upregulating expression of antioxidative Nrf2 target genes, such as *Ho-1* (*26*). In addition, we recently found that CA promotes neurite outgrowth of PC12h cells through Nrf2-dependent and -independent mechanisms (*27*).

Kosaka and Yokoi (*28*) reported that CA induces NGF production in T98G glioblastoma cells. However, the molecular mechanism by which CA enhances NGF production remains unknown. In the present study, we examined the role of the Nrf2 signalling pathway in CA-inducible *NGF* gene expression in T98G cells and normal human astrocytes (NHAs). Our finding that Nrf2 regulates *NGF* expression further supports targeting this transcription factor in the prevention and treatment of neurodegenerative diseases.

Materials and Methods

Materials

CA was provided from Nagase Co Ltd (Kobe, Japan). Dimethyl sulphoxide (DMSO), diethyl maleate (DEM), 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and human recombinant IL-1β were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Actinomycin D was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Human recombinant TNFα was obtained from PeproTech Inc. (Rocky Hill, NJ, USA). Anti-Nrf2 and anti-Keap1 rat monoclonal antibodies were described previously (*29*, *30*). Anti-Nrf2 (sc-13032) and anti-Lamin B (sc-6217) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-β-Actin (A1978) was purchased from Sigma-Aldrich. Minimal essential medium (MEM), Opti-MEM I reduced serum medium and foetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin (BSA) was obtained from Nacalai tesque (Kyoto, Japan). Normal human astrocytes (NHAs) and astrocyte growth medium (AGM) were obtained from Lonza Ltd. (Basel, Switzerland).

Cell culture

T98G human glioblastoma cells were maintained in MEM supplemented with NEAA, glutamine, pyruvic acid, penicillin, streptomycin and 10% FBS. T98G cells were derived from glioblastoma multi-forme that corresponded to a grade IV WHO astrocytoma classification (*24*). NHAs were maintained in AGM. Cells were maintained at 37°C in a 5% CO_2 incubator. For CA treatment, T98G cells were pre-cultured in Opti-MEM-0.5% BSA for 24 h before treatment and then exposed to 2–50 μM CA for the time indicated in the figure legend. NHAs received 10 μM CA or 100 μM DEM in AGM.

RNA preparation and RT–qPCR

Total RNAs from T98G cells or NHAs were isolated by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Then, 1 μg total RNA was reverse transcribed to cDNA using the PrimeScript cDNA synthesis kit (Takara Bio Inc., Otsu, Japan). An aliquot of cDNA was used for gene expression analysis with the TaKaRa SYBR green qPCR premix and Chromo 4 real-time PCR system. The primers used for RT–qPCR were: NGF (forward 5′-AGA GAG CGC TGG GAG CCG GAG-3′; reverse 5′-GGC AGT GTC AAG GGA ATG CTG AAG T-3′), BDNF (forward 5′-CAG AAT CGG AAC CAC GAT G-3′; reverse 5′-AAA AGG ATG GTC ATC ACT CTT CTC-3′), NT-3 (forward 5′-GCG ACA ACA GAG ACG CTA CA-3′; reverse 5′-CAC GTA ATC CTC CAT GAG ATA CAA-3′), HO-1 (forward 5′-CCA GCA ACA AAG TGC AAG ATTC-3′; reverse 5′-TCA CAT GGC ATA AAG CCC TAC AG-3′), TXNRD1 (forward 5′-ACA CAA AGC TTC AGC ATG TCA-3′; reverse 5′-CAA TTC CGA GAG CGT TCC-3′), rRNA (forward 5′-CGG CTA CCA CAT CCA AGG AA-3′; reverse 5′-GCT GGA ATTA CCG CGG CT-3′), Cyclophilin A (forward 5′-ATG CTG GAC CCA ACA CAA AT-3′; reverse 5′-TCT TTC ACT TTG CCA AAC ACC-3′).

Immunoblot analysis

T98G nuclear extracts or whole-cell lysates were resolved with 8% or 10% SDS–PAGE and then transferred onto PVDF membranes using the semi-dry electrotransfer method. The transferred membranes were blocked with 1% skim milk–PBS-0.1% Tween-20, blotted with anti-Nrf2 rat monoclonal antibody or anti-Nrf2 antibody (sc-13032) and anti-Lamin B or anti-β-actin antibodies and then visualized using ImmunoStar chemiluminescent reagent (Wako).

siRNA transfection

T98G cells were cultured in Opti-MEM-0.5% BSA for 24 h before transfection. Immediately before transfection, the medium was changed to Opti-MEM, and then siRNA against human Nrf2 or Keap1 or control siRNA (40 pmol/well) was delivered into the cells using Lipofectamine 2000 (Invitrogen). After a 4-h incubation, the medium was changed to Opti-MEM-0.5% BSA, and the cells were incubated for 20 h. Twenty-four hours after transfection, the transfected cells were treated with DMSO or 50 μM CA for 6 or 24 h for the whole-cell extract or RNA preparation, respectively. The siRNA target sequences against human Nrf2 and Keap1 were described previously (*31*, *32*).

Enzyme-linked immunoSorbent assay

NGF protein secretion into culture supernatant was evaluated using Emax ImmunoAssay System (Promega, WI, USA) according to the manufacturer's protocol.

Plasmid construction and transient transfection

A dominant negative Nrf2 mutant expression vector (pcDNA3-hNrf2 DN) was constructed by PCR cloning using PCR primers (hNrf2-fwd4: 5′-GCG GAT CCA CCA TGG CCC AAT GTG AGA ACA CAC CA-3′ and hNrf2-rev2: 5′-GCT CTA GAC ATT TCA CAT CAC AGT AGG AGC-3′) and human Nrf2 cDNA as a template. Amplified cDNA fragment was digested with BamHI and XbaI, and subcloned into BamHI/XbaI sites of pcDNA3. The cDNA sequence was confirmed by DNA sequencing. T98G cells were transfected with either pcDNA3 or pcDNA3-hNrf2 DN plasmid using FuGENE HD transfection reagent (Roche, Basel, Switzerland). After 24 h transfection, the media were changed to Opti-MEM-0.5% BSA containing DMSO or 50 μM CA, and then the cells were incubated for 6 h. RT–qPCR and immunoblot analysis was performed as described above.

Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. The difference between the averages of each group was determined by Student's *t*-test or one-way ANOVA with Turkey–Kramer multiple comparison test. $P<0.05$ was considered statistically significant.

Results

CA induces NGF mRNA expression

Our previous study demonstrated that CA induces NGF production in the T98G human glioblastoma cell line (*28*). To understand the mechanism behind CA-induced NGF production in T98G cells, we examined *NGF* mRNA expression in CA-treated T98G cells. As shown in Fig. 1A, *NGF* mRNA was gradually induced in a concentration-dependent manner up to 50 μM CA. Although we tested 100 μM CA, it induced cell death in T98G cells, and we mainly used 50 μM CA in subsequent experiments. A time course analysis revealed that CA induced *NGF* mRNA expression in a time-dependent manner, peaking at 24 h and decreasing at 48 h (Fig. 1B). On the other hand, the expression of other neurotrophins, BDNF and NT-3, was not induced in T98G cells by CA treatment (Fig. 1B). To investigate the effect of CA on *NGF* mRNA stability, we employed actinomycin D to halt gene transcription. As shown in Fig. 1C, CA did not substantially alter *NGF* mRNA stability (estimated half-life $t_{1/2}$ ~76.7 ± 6.60 min and 86.8 ± 5.76 min for DMSO- and CA treated, respectively). This observation suggests that CA increases *NGF* mRNA expression at the transcriptional level.

CA induces NGF in an Nrf2-dependent manner

We previously reported that CA activates the Nrf2 signalling pathway by increasing Nrf2 nuclear accumulation in rat pheochromocytoma PC12h cells (*26*, *27*). CA also activates the Nrf2 pathway in the human neuronal cell line HT22 (*33*). To investigate whether CA activates Nrf2 signalling in T98G cells, we analysed the nuclear accumulation of Nrf2 after CA treatment. As shown in Fig. 2A, Nrf2 protein accumulation in the nuclear fraction was observed 2 h after CA treatment and continued until the 24-h time point. Nuclear accumulation of Nrf2 was also confirmed by immunocytochemical analysis (Supplementary Fig. S1A). Consistent with these observations, CA induced mRNA expression of typical Nrf2-targeted genes such as *HO-1*, *TXNRD1*, *GCLC* and *SQSTM1* in

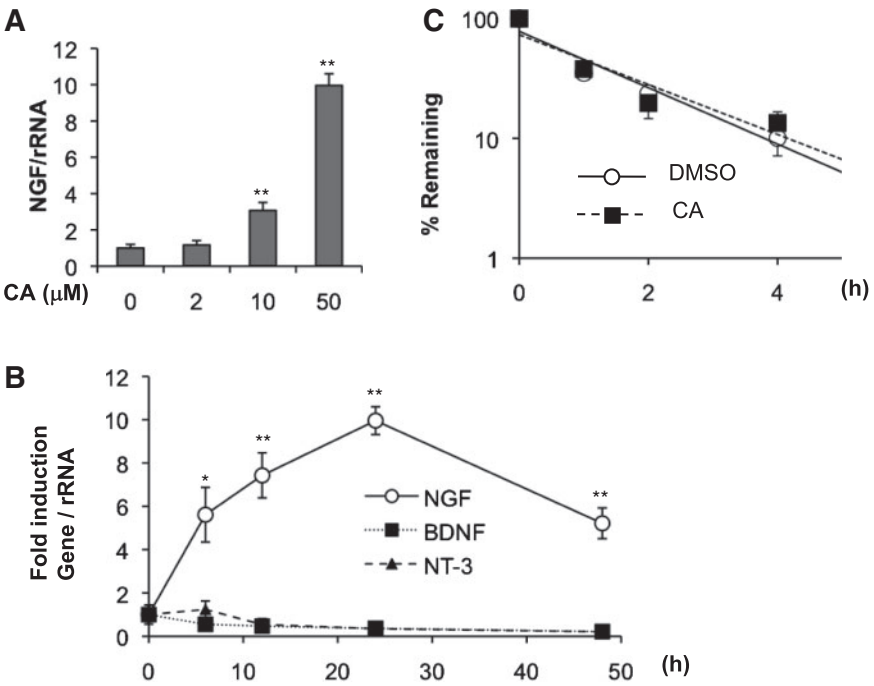

Fig. 1 CA induces *NGF* mRNA expression in T98G cells. (A) T98G cells were treated with 2–50 μM CA for 24 h, and then *NGF* gene expression was measured by RT–qPCR. *NGF* gene expression was normalized with *rRNA* expression. (B) T98G cells were exposed to 50 μM CA for the time indicated in the figure. *NGF* (open circle, solid line), *BDNF* (closed square, dotted line) and *NT-3* (filled triangle, dashed line) gene expression levels were evaluated as described in (A). (C) T98G cells were treated with DMSO (open circle, solid line) or 50 μM CA (closed square, dashed line) in combination with 1 μg/ml actinomycin D for 1–4 h, and *NGF* transcripts were measured by RT–qPCR. *NGF* gene expression was normalized with cyclophilin A expression. Values are the average of at least three independent experiments and presented as a fold of 0 h or the DMSO control (= 1). Error bars indicate SEM. *$P<0.05$; **$P<0.01$.

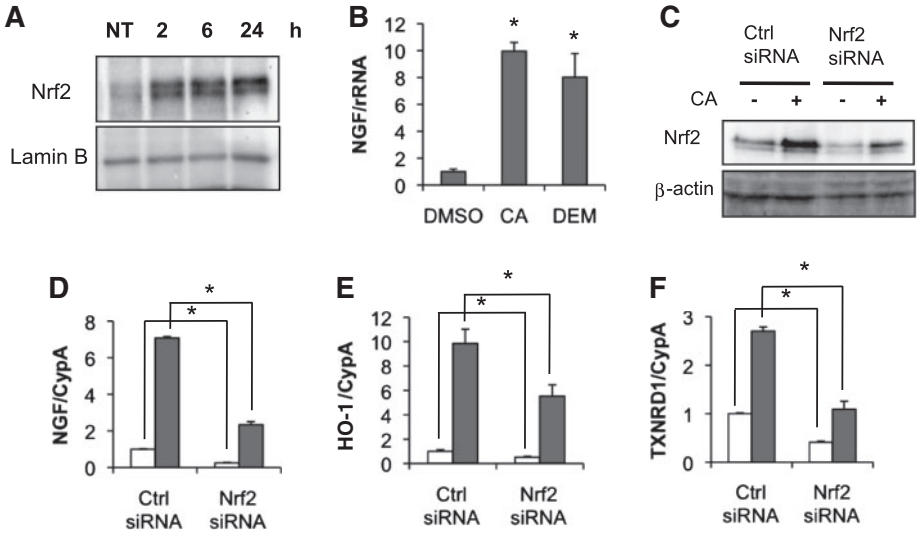

Fig. 2 Nrf2-dependent induction of *NGF* mRNA in CA-treated T98G cells. (A) T98G cells were treated with 50 μM CA for 2–24 h, and nuclear extracts were subsequently prepared as described in the 'Materials and Methods' section. An aliquot of nuclear extracts was separated by SDS-PAGE and blotted with anti-Nrf2 or Lamin B antibodies. (B) T98G cells were treated with either DMSO, 50 μM CA or 100 μM DEM for 24 h, and then *NGF* mRNA expression was measured by RT–qPCR analysis. Gene expression levels were normalized with rRNA expression. Values are the average of at least three independent experiments and presented as a fold of DMSO (= 1). Error bars indicate SEM. $*P<0.01$ (versus DMSO). (C) Immunoblot analysis of siRNA-transfected cells. T98G cells were transfected with either control (Ctrl siRNA) or anti-Nrf2 siRNA (Nrf2 siRNA). siRNA-transfected cells were exposed to DMSO or 50 μM CA for 6 h. An aliquot of whole-cell lysates was separated by SDS–PAGE and then blotted with ani-Nrf2 and anti-β-actin antibody. (D–F) T98G cells were transfected with either control (Ctrl siRNA) or anti-Nrf2 siRNA (Nrf2 siRNA). Twenty-four hours after siRNA transfection, the cells were exposed to DMSO (open bar) or 50 μM CA (closed bar) for an additional 24 h. Total RNA was then subjected to RT–qPCR analysis to measure *NGF* (D), *HO-1* (E) and *TXNRD1* (F) gene expression. The value of the DMSO- and control siRNA-treated cells was arbitrarily set as 1 and relative expressions were expressed as the means of at least three independent experiments with the SEM. $*P<0.01$.

T98G cells (Supplementary Fig. S1B). In addition, other type of Nrf2 inducers, such as diethyl maleate (Fig. 2B) and sulphoraphane (data not shown) also induced *NGF* mRNA expression suggesting the involvement of Nrf2 signalling pathway in CA-inducible *NGF* expression. To clarify the necessity of Nrf2 in CA-induced *NGF* expression, we knocked down endogenous Nrf2 using siRNA against Nrf2. Depletion of the Nrf2 protein by siRNA was confirmed by immunoblot analysis (Fig. 2C). As expected, *NGF* mRNA induction by CA was decreased by ~40% similarly as other Nrf2 target genes *HO-1* and *TXNRD1* by Nrf2 knockdown (Fig. 2D–F). Although the suppression of Nrf2 activity may perturb cellular redox balance (*14*, *15*), Nrf2 knockdown did not significantly affect T98G cell viability (Supplementary Fig. S2A). In addition, constitutive expression of *NGF* was also decreased by Nrf2 knockdown (Fig. 2D). The similar result was obtained by using alternative anti-Nrf2 siRNA (Supplementary Fig. S2B). These results indicate that Nrf2 is indispensable for constitutive and CA-inducible *NGF* expression.

NGF is induced through Nrf2 signalling pathway in NHAs

To evaluate the role of Nrf2 signalling pathway in the *NGF* gene regulation in non-transformed astrocytes, we examined the *NGF* inducing activity in NHAs. In NHAs, CA induced *NGF* gene expression and NGF protein secretion into culture medium (Fig. 3A and B). DEM also substantially induced *NGF* gene expression and NGF protein production although the effect did not reach statistical significance (data not shown). The knockdown of endogenous Nrf2 by siRNA in NHAs attenuated CA-inducible *NGF* gene expression and NGF protein production as in T98 cells (Fig. 3C and D). Depletion of the Nrf2 protein by siRNA was confirmed by immunoblot analysis (Fig. 3E). These results indicate that Nrf2 signalling pathway modulates *NGF* gene expression in NHAs.

Nrf2 regulates NGF gene expression distinctively from typical Nrf2 target genes in T98G cells

To investigate whether Nrf2 activation is sufficient to induce *NGF* expression, the Nrf2 inhibitor Keap1 was knocked down by siRNA. Keap1 knockdown enhanced Nrf2 protein accumulation without CA treatment in T98G cells (Fig. 4A). As shown in Fig. 4B, Keap1 knockdown enhanced constitutive *NGF* expression, and CA treatment further induced *NGF* expression. Keap1 knockdown did not affect T98G cell viability and the similar result was obtained using alternative anti-Keap1 siRNA (Supplementary Fig. S2A and B). However, Keap1 knockdown did not activate constitutive expression of *HO-1* or *TXNRD1*; surprisingly, their CA-inducible expressions were decreased compared with their expressions in control siRNA-treated cells (Fig. 4C). These results indicate that the regulatory mechanism of *NGF* gene induction by Nrf2 is different from the expression of other Nrf2 target genes. To clarify the role of Nrf2 in Keap1 knockdown-induced *NGF* gene up-regulation, we simultaneously knocked down both Keap1 and

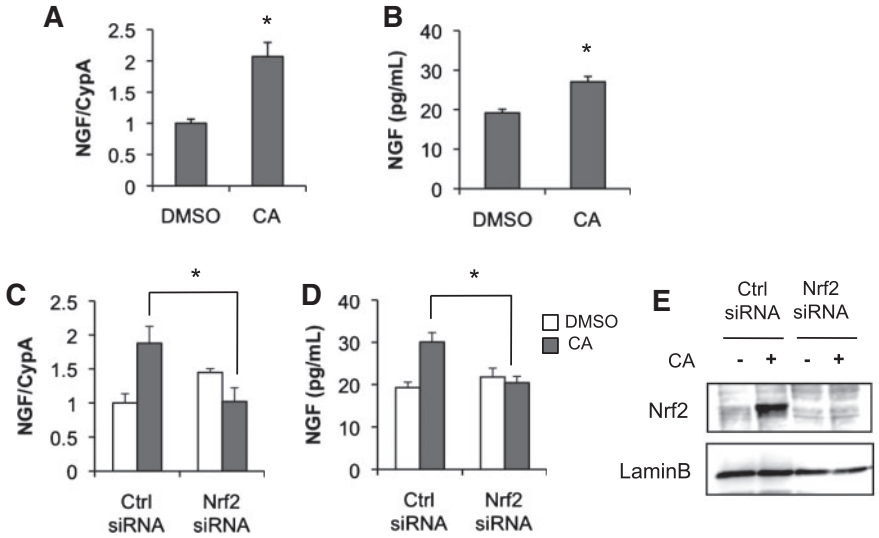

Fig. 3 CA induces *NGF* gene expression in normal human astrocytes. (A and B) NHAs were exposed to DMSO or 10 μM CA for 24 h. *NGF* gene expression was measured by RT–qPCR (A), and NGF protein secretion into culture media was analysed by ELISA as described in 'Materials and Methods' section (B). (C and D) NHAs were transfected with control (Ctrl siRNA) or anti-*Nrf2* siRNA (Nrf2 siRNA) and then administrated with DMSO or 10 μM CA for 24 h. *NGF* gene expression (C) and protein secretion (D) in knocked down cells were measured as described above. Values are the average of at least three independent experiments and presented as fold of the DMSO control or control siRNA (DMSO) for RT–qPCR analysis. Error bars indicate SEM. $*P<0.01$. (E) Immunoblot analysis of control or Nrf2 siRNA transfected NHAs.

Nrf2. Immunoblot analysis showed Keap1 knockdown-induced Nrf2 protein accumulation was diminished by Nrf2 siRNA (Fig. 4D). Correspondingly, Keap1 knockdown-provoked *NGF* induction was attenuated by additional Nrf2 knockdown, although the *NGF* expression level was higher than in cells where only Nrf2 was knocked down by siRNA (Fig. 4E). Interestingly, the over-expression of a dominant negative Nrf2 mutant (DN-Nrf2), which lacks the N-terminal transactivation domain of Nrf2 (*i.e.* amino acids 1–419) did not affect CA-inducible *NGF* expression in T98G cells, although *HO-1* expression was significantly attenuated (Fig. 5A). Immunoblot analysis revealed that CA comparably induced the protein expression of endogenous Nrf2 in between vector- and DN-Nrf2-transfected cells (Fig. 5B). These results indicate that Nrf2 mediates the *NGF* induction provoked by Keap1 knockdown but that the *NGF* induction mechanism is distinct from typical Nrf2 target genes *HO-1* and *TXNRD1* in T98G cells.

Nrf2 signalling cooperates with other NGF inducing stimuli

TPA, TNFα and IL-1β are reported to induce *NGF* gene expression in various cell types (*8*, *11*, *12*). In T98G cells, IL-1β induced *NGF* expression as potently as CA (~9.1-fold), and TPA and TNF-α-induced *NGF* expression to a lesser extent (~2.0- and ~3.3-fold, respectively) (Fig. 6A). Intriguingly, CA and IL-1β synergistically enhanced *NGF* gene expression, whereas TPA or TNF-α additively enhanced CA-inducible *NGF* gene expression (Fig. 6A). To elucidate the role of Nrf2 signalling pathway in TPA-, TNF-α and IL-1β-inducible *NGF* expression, we next knocked down Nrf2. Nrf2 knockdown decreased constitutive and TPA- or TNF-α-inducible *NGF* expression, while IL-1β-induced *NGF* induction was unaffected (Fig. 6B). These results indicate that Nrf2 signalling pathway and other NGF inducing stimuli co-ordinately modulate *NGF* expression in T98G cells.

Discussion

In this study, we demonstrated that CA up-regulates *NGF* mRNA levels without affecting the mRNA stability (Fig. 1). Therefore, we speculate that CA-induced *NGF* mRNA expression is transcriptional. To elucidate whether Nrf2 directly enhances *NGF* gene expression, we performed a luciferase-based reporter assay. However, we failed to detect CA- or Nrf2-inducible reporter gene expression using *NGF* gene promoter-luciferase constructs (up to −16 kb of the human *NGF* gene promoter region) (data not shown). We are now trying to find the CA- and Nrf2-responsible regions in the *NGF* gene locus.

NGF production is regulated through multiple signalling pathways, and several transcription factors are thought to activate *NGF* gene induction. TPA enhances *NGF* gene transcription through the AP-1 site in the first intron of the *NGF* gene (*7*, *8*). The same AP-1 site is also responsible for *NGF* induction by $1,25(OH)_2D_3$ and isoproterenol, a microsomal Ca^{2+}-ATPase inhibitor (*34*, *35*). Because TPA and CA additively enhance *NGF* gene expression (Fig. 6), CA and $1,25(OH)_2D_3$ or isoproterenol may also cooperate in *NGF* gene regulation. A dopamine agonist, bromocriptine and a cyclopentenone prostaglandin, 15 d-PGJ_2, also induce *NGF* mRNA expression in cultured mouse cortical astrocytes (*36*, *37*). Curiously, both bromocriptine and 15 d-PGJ_2 function as Nrf2 inducers (*38*, *39*). Therefore, we speculate that Nrf2 may be involved in *NGF* gene induction by these chemicals, although bromocriptine and 15d-PGJ_2 induce

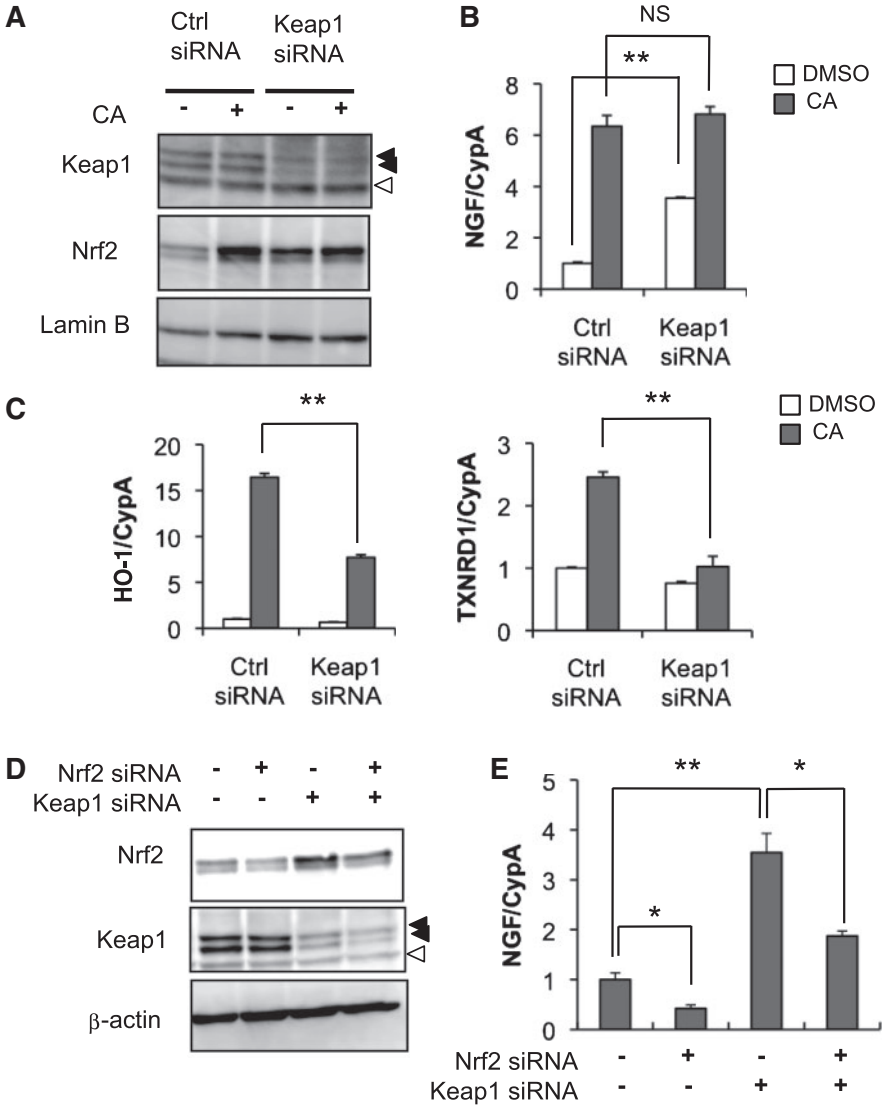

Fig. 4 Keap1 knockdown enhances Nrf2 accumulation and *NGF* gene expression. (A and D) Immunoblot analysis of siRNA-transfected cells. T98G cells were transfected with control siRNA (Ctrl siRNA), anti-Keap1 siRNA (Keap1 siRNA) or anti-Nrf2 siRNA (Nrf2 siRNA) and then exposed to DMSO or 50 μM CA for 6 h. An aliquot of whole-cell lysates was separated by SDS–PAGE and then blotted either with anti-Keap1, anti-Nrf2, anti-β-actin or anti-Lamin B antibodies. Closed and open triangles indicate Keap1 and a non-specific band, respectively. (B and C) T98G cells were treated with control or Keap1 siRNA, and then *NGF* (B) or *HO-1* and *TXNRD1* (C) gene expression was analysed by RT–qPCR. (E) *NGF* gene expression in Nrf2 and Keap1 double knockdown cells was analysed by RT–qPCR. The value of the DMSO- and ctrl-siRNA-treated cells was arbitrarily set as 1 and relative expressions were expressed as the means of at least three independent experiments with the SEM. $*P<0.05$; $**P<0.01$; NS, not significant.

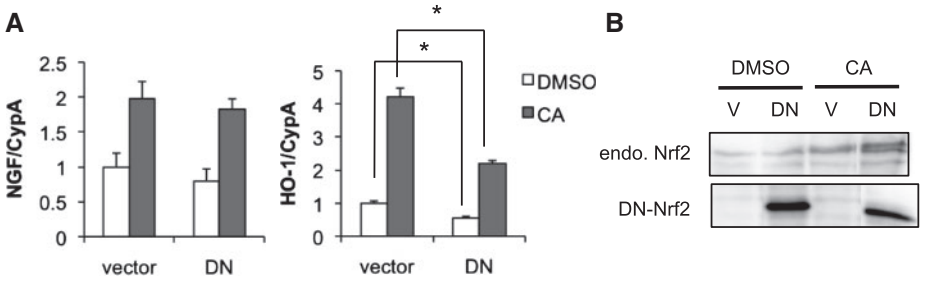

Fig. 5 Effect of a dominant negative Nrf2 mutant on CA-inducible *NGF* gene expression. (A) T98G cells were transfected with vacant vector (V) or dominant negative Nrf2 mutant expression vector (DN). The transfected cells were treated with DMSO (open bar) or 50 μM CA (closed bar) for 6 h, and then *NGF* or *HO-1* gene expression was measured by RT–qPCR analysis (normalized with cyclophilin A expression). The value of the DMSO- and vector-treated cells was arbitrarily set as 1 and relative expressions were expressed as the means of at least three independent experiments with the SEM. $*P<0.01$. (B) T98G cells were transfected and treated as in (A) and the protein expression of endogenous Nrf2 (endo. Nrf2) or transfected dominant negative Nrf2 mutant (DN-Nrf2) was analysed by immunoblot analysis using the whole-cell extracts.

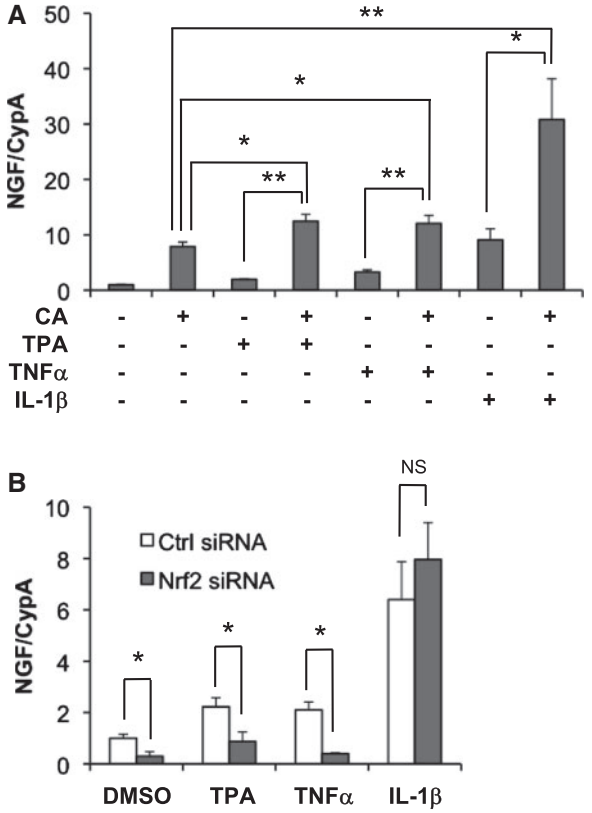

Fig. 6 Nrf2 cooperates with NGF inducing stimuli. (A) T98G cells were untreated or treated with either 100 ng/ml TPA, 10 ng/ml hIL-1β or 10 ng/ml hTNFα alone or in combination with 50 μM CA. (B) T98G cells were transfected with control or Nrf2 siRNA as described in 'Materials and Methods' section, and 24 h after transfection, transfected cells were administrated with DMSO, 100 ng/ml TPA, 10 ng/ml TNFα or 10 ng/ml IL-1β for additional 24 h. *NGF* gene expression was analysed by RT–qPCR. *NGF* gene expression was normalized with cyclophilin A expression. The value of the DMSO-treated cells was arbitrarily set as 1 and relative expressions were expressed as the means of at least three independent experiments with the SEM; $*P<0.05$; $**P<0.01$; NS, not significant.

BDNF mRNA in mouse cortical astrocytes, but CA did not induce its expression in T98G cells (Fig. 1B).

NGF functions as an inflammatory mediator in both neuronal and non-neuronal tissues (*40*, *41*). Accordingly, high NGF expression has been observed in various inflammatory diseases (*41*). Interestingly, NGF and TNF-α are proposed to constitute a cytokine network in CNS (*40*). TNF-α increases NGF production in glia and immune cells (*11*, *12*, *40*). Conversely, NGF increases TNF-α production in neurons, and TNF-α promotes neuronal cell survival via TNFR2 by co-operatively activating the Akt-mediated survival signal with NGF or apoptosis when NGF is absent. Therefore, this positive feedback loop between NGF and TNF-α likely decreases neuronal cell death during inflammation. In T98G cells, both IL-1β and TNF-α exerted cooperative effect with CA on the induction of *NGF* expression (Fig. 6A). In addition, TNFα-inducible *NGF* expression was attenuated by Nrf2 knockdown (Fig. 6B). Therefore, although it has yet to be determined whether glial cells or immune cells in the CNS actually produce NGF through CA or during inflammation in an Nrf2-dependent manner, Nrf2 may play an important role in the CNS NGF-inflammatory cytokine network, and CA may decrease neuronal cell death during inflammation.

Previous reports demonstrated that Keap1 ablation results in constitutive Nrf2 activation and target gene induction (*30*, *32*, *42–44*). In T98G cells, Keap1 knockdown resulted in Nrf2 accumulation. Immunocytochemical and subcellular fractionation analyses revealed that Nrf2 accumulated in the nuclei in Keap1 knockdown cells, as observed in CA-treated cells (Fig. 4; our unpublished observation). However, Keap1 knockdown in T98G cells did not increase *HO-1* and *TXNRD1* expression, but surprisingly attenuated CA-induced *HO-1* or *TXNRD1* expression. These results suggest that CA-inducible expression of *HO-1* and *TXNRD1* requires Keap1, and Nrf2 nuclear accumulation alone is not sufficient to induce the prototypical Nrf2 target genes in T98G cells. On the other hand, Keap1 knockdown up-regulated constitutive *NGF* mRNA expression, and CA treatment further increased its expression. Simultaneous knockdown of Nrf2 and Keap1 attenuated the *NGF* induction elicited by Keap1 knockdown alone (Fig. 4). Although *NGF* expression in Keap1/Nrf2 double knockdown cells was higher than that in Nrf2 knockdown cells, *NGF* expression levels were correlated with the Nrf2 protein levels elicited by the respective siRNA treatments (Fig. 4D). In addition, the over-expression of DN-Nrf2 did not affect CA-inducible *NGF* expression (Fig. 5). These results suggest that both *NGF* and other Nrf2 target gene (*HO-1* and *TXNRD1*) expressions commonly require Nrf2 but point to the distinct regulatory mechanisms involved.

In this article, we demonstrated a role of Nrf2 in CA-inducible *NGF* gene expression in T98G glioblastoma cells and in NHAs. The results are consistent with our recent observation that Nrf2 is also involved in the CA-inducible *NGF* expression in human astrocytoma U373MG cells (*45*). Thus, in addition to yet unknown roles of Nrf2 in astrocytoma/glioblastoma cells, we speculate that Nrf2 may play a physiological role in astrocytes to up-regulate NGF expression. Specific Nrf2 over-expression in astrocyte of mice confers neuroprotection against various types of neurodegenerative diseases (*46*, *47*). Although its mechanisms are largely unknown, secretable factors are postulated that transmit surviving signals from astrocytes to neurons in an Nrf2-dependent manner. An example of such factor is glutathione. Nrf2 regulates glutathione synthesis in astrocytes (*48*, *49*) and glutathione secretion from astrocytes may confer neuroprotection by attenuating oxidative stress. We surmise that NGF can be another Nrf2-dependent neuroprotective factor secretable from astrocytes. Interestingly, we recently demonstrated that Nrf2 induces neurite extension and that NGF activates the Nrf2 signalling pathway in rat neuron-like PC12h cells (*28*). Therefore, we surmise that Nrf2 in both neurons and astrocytes may strengthen NGF function in the brain to regulate neuronal differentiation and plasticity as well as the neuroprotective response.

In conclusion, we demonstrated here that CA stimulates *NGF* gene expression through an Nrf2-dependent pathway. Although the precise mechanism of Nrf2-dependent induction of the *NGF* gene is still unclear, Nrf2-mediated NGF induction may be involved in the protection of neurons during inflammation and also in physiological processes in the brain such as neuronal plasticity. These possibilities remain open to future studies.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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